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(54) Title: POLYANIONIC POLYAMINO ACID INHIBITORS OF MINERAL DEPOSITION AND THEIR SYNTHESIS (57) Abstract Polypeptides of the formula poly (X) _m (Y) _n , wherein X is selected from the group consisting of aspartate, glutamate, glutamine, asparagine, mixtures and block polymers thereof, Y is selected from the group consisting of phosphoserine, sulfo-serine, phosphohomoserine, phosphotyrosine, phosphotreonine, phosphoglutamine, phosphoasparagine, serine, alanine, dehydroalanine and mixtures thereof, m = 5-199 and n = 1-10 have been demonstrated to <u>inhibit mineral deposition</u> , and can be employed in the inhibition of mineral deposition in both <i>in vitro</i> and <i>in vivo</i> applications. Serine-containing polypeptides can be thermally synthesized by combining the necessary amino acid residues in a reaction vessel, eliminating oxygen to preclude charring, and heating said reaction vessel in a heating medium to a temperature of about 155 °C.		

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DescriptionPolyanionic Polyamino Acid Inhibitors Of
Mineral Deposition And Their SynthesisTechnical Field

5 This invention relates to the discovery of new
polyamino acids and their synthesis. The polyamino acids
are powerful inhibitors of mineral formation,
particularly the crystallization of calcium carbonate and
calcium phosphate. The molecules may be useful in a
10 variety of applications including but not limited to
tartar control, prevention of industrial scaling, use as
dispersants, corrosion inhibition, prevention of
pathological calcification, and control of biofouling.

Background Art

15 Biological mineralization is a fundamental process
in nature. Unfortunately mineral deposits frequently
occur where they are not wanted. In the body, mineral
deposition may contribute to dental plaque, hardening of
the arteries, various organ stones and the failure of
20 prosthetic devices like implanted heart valves. In the
marine environment, the biomineral structures may cause
problems as in the case of barnacles growing on the hulls
of ships, adding extra bulk and creating drag. In
industry, mineral scale forms on surfaces of cooling
25 towers and other devices preventing their proper
operation as heat exchangers and frequently promoting
localized corrosion.

Because of the problems associated with unwanted
mineral deposition, much effort has been devoted to

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finding mineralization inhibitors, particularly in industry, that might be used to prevent harmful mineral formation.

Among the newest approaches to developing mineral
5 deposition inhibitors is the study of naturally-occurring proteins and polysaccharides that regulate mineral formation by organisms (Sikes, C.S. and A.P. Wheeler (1985). Inhibition of inorganic or biological CaCO_3 deposition by polyamino acid derivatives. U.S. Patent
10 4,534,881; Sikes, C.S. and A.P. Wheeler (1986). Inhibition of inorganic and biological CaCO_3 deposition by a polysaccharide fraction obtained from CaCO_3 -forming organisms. U.S. Patent 4,585,560; Wheeler, A.P. and C.S. Sikes (1986). Inhibition of the formation of inorganic
15 or biological CaCO_3 -containing deposits by a proteinaceous fraction obtained from CaCO_3 -forming organisms. U.S. Patent 4,587,021). This approach led to the identification of a new class of polyanionic/hydrophobic peptides (Sikes, C.S. and A.P.
20 Wheeler (1989). Inhibition of mineral deposition by polyanionic/hydrophobic peptides and derivatives thereof having a clustered block copolymer structure. U.S. Patent 4,868,287) and phosphorylated and related polyanionic peptides (Sikes, C.S. Inhibition of mineral
25 deposition by phosphorylated and related polyanionic peptides. U.S. Patent application 07/339,672) that are even more powerful inhibitors of crystallization on a weight basis than the natural proteins. The preferred structure of the phosphopeptides is separated as
30 $\text{Asp}_{(x)}\text{P Ser}_{(y)}$ with $x = 10$ to 40 and $y = 1$ to 3 .

Solid phase synthesis techniques described to make

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these peptides are presently thought to be commercially non-viable due to the costs involved. Now the inventors have discovered new polyamino acids, including a cost effective method for their bulk synthesis.

5 Clues for development of this method were taken from the observation that mixtures of α -amino acids could be thermally polymerized into proteinoids (Fox, S.W. and K. Harada (1958). Thermal copolymerization of amino acids to a product resembling protein. Science, 128, 1214;
10 Fox, S.W. and K. Harada (1959). The thermal copolymerization of amino acids common to protein. J. Am. Chem. Soc., 82, 3745-3751) at temperatures above 150°C. The synthesis required the presence of excess dicarboxylic amino acids for unknown reasons. It was
15 further noted that there was a high reproducibility in the copolymerization of amino acids (Fox, S.W. and C.R. Windsor (1984). Reproducibility of amino acid compositions in repeated copolymerizations of amino acids. Int. J. Quart. Biol. Symp., 11, 103-108). This
20 suggested a possible simple method for preparing mineral deposition inhibitors which are composed primarily of the dicarboxylic amino acid aspartate.

However, the advantage of excess dicarboxylic amino acid was reportedly lost above 210°C, with thermal
25 decomposition of the amino acids. A further problem was that incorporation of serine into proteinoid was generally not possible due to thermal decomposition even at relatively low temperatures. When serine was incorporated to a limited extent under mild conditions,
30 yields were quite low (Fox, S.W. and K. Harada (1960). Thermal copolymerization of amino acids common to

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protein. J. Am. Chem. Soc., 87, 3745-3751; Fox, S.W., K. Harada and D.L. Rohlfing (1962). The thermal copolymerization of α -amino acids, in: M.A. Stahmann (ed.), Polyamino Acids, Polypeptides, and Proteins.

5 Univ. of Wisconsin Press, Madison, pp. 47-54).

The inventors now report methods of increasing the incorporation of serine into thermal polyamino acids. The effects of varying the composition and ratios of reactants, solvent systems, temperature, and time of
10 synthesis were examined.

In short, the present invention identifies new polyanionic polypeptide molecules and a method of synthesis. The molecules preferably contain a polyaspartate backbone with phosphoserine or a derivative
15 thereof incorporated into the polymer. Sulfated, phosphonated and sulfonated derivatives of serine and other amino acids are also contemplated for incorporation into the aspartate backbone. Such polypeptides of this invention and the method of synthesis have not been
20 specifically described before. Additionally, the method of synthesis has not been described previously with the exception of thermal polyaspartic acid.

Disclosure of the Invention

Accordingly, it is an objective of the present
25 invention to provide new and improved materials for inhibiting mineral deposition.

It is another objective of this invention to provide a method for bulk synthesis of these materials.

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It is another objective of the present invention to provide materials for prevention of formation of calculus or plaque on teeth.

5 It is another objective of the present invention to provide materials that can prevent mineralization in a marine environment, such as by prevention of barnacle accumulation.

10 It is another objective of the present invention to provide materials that can effectively prevent mineralization of prosthetic devices implanted in the body.

15 It is another objective of the present invention to provide materials for prevention of mineralization in arteries associated with arteriosclerosis, or atherosclerosis.

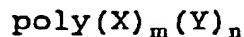
It is another objective of the present invention to provide materials that can prevent mineralization in an industrial setting, such as scaling in cooling towers and boilers.

20 It is another objective of the present invention to provide for materials that can prevent corrosion of metallic surfaces.

25 These and other objectives of the present invention which will hereinafter become more readily apparent have been accomplished by providing new polypeptide materials that have the following general formula. The materials consist of a block of anionic amino acids connected to

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another block that contains anionic derivatives of hydroxylated or aminated amino acids mixed with hydrophobic residues.



5 where each X is aspartate, glutamate, glutamine, asparagine, or anionic derivatives of these amino acids or a block polymer thereof.

10 each Y is an anionically derived amino acid such as phosphoserine, sulfoserine, phosphohomoserine, phosphotyrosine, phosphothreonine, phosphoglutamine, phosphoasparagine or mixtures of these residues; serine; alanine, or
15 dehydroalanine.

m = 5 to 199

n = 1 to 40

It should be noted that occasional random monomers may appear in a short sequence, or between blocks, but
20 the molecule is substantially characterized as a block copolymer.

These polypeptides may be prepared by thermal polymerization of, e.g., polyaspartic acid, aspartic acid and serine. The selected amino acids are placed in a
25 reaction container, such as an Erlenmeyer flask. The reaction vessel is partially submerged in a heating medium, such as cooking oil, and heated to a temperature

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below the decomposition temperature of serine. An effective reaction temperature is $155^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Oxygen is eliminated from the reaction vessel to preclude charring by, e.g., continuously purging the reaction vessel with a stream of nitrogen. The reaction may be continued for up to 24 hours. If necessary, the product is purified by dissolving it in distilled water, and dialyzing it against the same to remove any unreacted amino acids. For some polypeptides, to achieve sufficient size, a starting backbone of thermally polymerized polyaspartate is required. Pre-existing polyaspartate can be introduced into the reaction vessel with serine, the pre-existing polyaspartate being prepared by separate temperature synthesis, either low or high temperature. In low temperature synthesis, aspartic acid is placed in a reaction vessel entrained in a heating medium such as cooking oil at about 190°C . Oxygen is purged, using a stream of nitrogen, to preclude charring. The reaction process may continue up to 72 hours. In high temperature synthesis, the aspartic acid is placed in a baking dish, and heated at 240°C in a muffle oven for up to 8 hours. Nearly 100% of the aspartic acid polymerized, requiring no further purification. High temperature thermally synthesized polyaspartate exhibits greater activity when compared to low temperature thermally synthesized polyaspartate, as an inhibitor of mineral formation.

The present invention is also directed to compositions containing these materials, such as dentifrices and mouthwashes for oral application.

Best Mode for Carrying Out the Invention

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In a preferred embodiment X is aspartate or glutamate, particularly preferably aspartate and most preferably polyaspartate of 10 to 60 amino acid residues.

5 The preferred amino acid composition of one peptide is 1 to 10 phosphoserines most preferably 3 to 6 with either derivatives of serine comprising approximately 2 to 5 residues of the molecule. The remainder of the polypeptide is aspartic acid preferably present as a block polymer of 40 to 60 residues.

10 The preferred amino acid composition of another peptide is 1 to 10 residues of alanine which result from serine decomposition; 1 to 12 residues of other derivatives of serine such as dehydroalanine most preferably 3 to 6; 0 to 2 residues of serine and the
15 remainder polyaspartic acid most preferably as a block polymer of 40 to 60 residues.

The preferred amino acid composition of another peptide is between 2 and 10 residues of derivatives of serine such as alanine and dehydroalanine most preferably
20 5 to 7 and the remainder of the peptide is 20 to 40 residues of aspartic acid.

Methods of Synthesis

This invention relates to methods of synthesis of the above described polypeptides. As solid phase methods
25 previously described for the synthesis of mineral deposition inhibitors (Sikes, C.S. Inhibition of mineral deposition by phosphorylated and related polyanionic peptides. U.S. Patent application 07/339,672) are

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expensive, cost effective methods for bulk synthesis were developed. Peptides of desired sequence and size may be made by thermal polymerization of amino acids.

- For example a mixture of thermally polymerized
- 5 polyaspartic acid, aspartic acid, and serine were placed in an Erlenmeyer flask. The reaction vessel was partially submerged in cooking oil heated to 155°C ($\pm 2^\circ\text{C}$). A stream of nitrogen was continuously purged into the reaction vessel to eliminate O_2 and the
- 10 possibility of charring. The reaction was allowed to continue for up to 24 hours. The product is purified by dissolving it in distilled water and dialyzing it against the same to remove any unreacted amino acids.

Detailed Description of Preferred Methods

- 15 For some polypeptides a starting backbone of thermal polyaspartate is required to provide sufficient size of the final product, given the relatively low reaction temperatures. Temperatures above 160°C led to decomposition of serine. Even at 155°C, an excess of
- 20 free aspartic acid was required for the serine to survive. Under these specific conditions, which were heretofore undescribed in the art, enough serine molecules become incorporated into the preexisting polyaspartate to produce the molecules of the invention.
- 25 The polyaspartate backbone can be prepared in one of two ways as detailed below.

Low Temperature Synthesis of Polyaspartate

This method is described in Sikes, C.S. Inhibition

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of mineral deposition by phosphorylated and related polyanionic peptides. U.S. Patent application 07/339,672. The entire disclosure of this application is incorporated herein by reference.

5 L-aspartic acid (500 g) was placed in a two-liter, round-bottom reaction vessel. The reaction vessel was partially submerged in cooking oil at 190°C ($\pm 2^\circ\text{C}$). The reaction vessel was coupled by ground glass fitting to a condenser vessel, which in turn was fitted to a rotator
10 shaft driven by a rheostated electric motor. A stream of nitrogen was continuously purged into the condenser vessel to eliminate O_2 and the possibility of charring. The reaction was allowed to continue up to 72 hours. Polyanhydroaspartic acid molecules of approximately 2500
15 daltons (determined by gel permeation) were produced. They were purified by dissolving at pH 8 in water followed by dialysis to remove unreacted aspartic acid although the bulk product is also usable without further purification.

20 High Temperature Synthesis of Polyaspartate:

L-aspartic acid (500g) was placed in a Pyrex baking dish and heated at 240°C in a muffle oven for up to 8 hours, preferably 6 hours. This resulted in nearly 100% of the aspartic acid being polymerized and no further
25 purification was necessary. Polyanhydroaspartic acid molecules of approximately 6000 daltons (determined by gel permeation) were produced. This molecule itself unexpectedly showed greatly increased activity over the low temperature thermal polyaspartate as an inhibitor of
30 mineral formation.

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Synthesis Example of Preferred SampleExample 1

Polyanhydroaspartic acid (9.7g, 0.1 mole) was hydrolyzed to polyaspartic acid by suspension in distilled water, pH 10, heated to 60°C for 1 hour and then neutralized with 10 N HCl. Small amounts of 10 N NaOH were added during the hour to maintain the pH at 10. The solution of polyaspartate was dried in the reaction vessel at 120°C for up to 1 hour under nitrogen. This was necessary in that water prevents peptide bond formation. L-serine and (2.62g, 0.025 mole) L-aspartic acid (6.65g, 0.05 mole) were then added and the temperature was brought up to 155°C ($\pm 2^\circ\text{C}$). This reaction was allowed to proceed for up to 24 hours, most preferably 8 to 10 hours. Longer reaction times resulted in total decomposition of serine. The molar ratio of amino acids mixed for the reaction was preferably 4:2:1, polyaspartate: aspartate: serine. The beginning polyanhydro aspartic acid was most preferably the high temperature form. The product consisted of 1 to 10 serine residues, most preferably 4 to 7. Derivatives of serine, such as alanine and dehydroalanine, are also present in amounts ranging from 1 to 10 residues.

Example 2

Orthophosphoric acid (Fox, S.W. and K. Harada (1960). Thermal copolymerization of amino acids common to protein. J. Am. Chem. Soc., 87, 3745-3751) has been found to be an effective solvent in the thermal synthesis of polyamino acids. It may act as a dehydrating agent or

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as an acid catalyst. It may also function by producing phosphorylamino acid intermediates, which are mixed acid anhydrides (Fox, S.W. and K. Harada (1958). Thermal copolymerization of amino acids to a product resembling protein. Science, 128, 1214).

Polyanhydroaspartic (9.7g) acid was converted to polyaspartate and neutralized as above. This solution of polyaspartate was dried as above and L-serine (2.62g), L-aspartic acid (6.65g), and 10 ml phosphoric acid (H_3PO_4 , 85 wt%) were added and the temperature was brought up to 155°C ($\pm 2^\circ\text{C}$). This reaction was allowed to proceed for up to 10 hours, preferably 6 hours. The molar ratio of polyaspartate: aspartate: serine was 4:2:1 and the moles of H_3PO_4 added were from 0.01 to 0.1 preferably 0.02 to 0.04. Phosphoric acid destroys most of the serine so that the final product had less than 1 serine residue/molecule but conversion products of between 1 and 10 alanine residues and 1 to 10 dehydroalanine. The polypeptide showed surprising activity, significantly increased relative to polyaspartate, especially in the inhibition of calcium phosphate formation.

Example 3

L-aspartic acid (13.34g, 0.1 mole) and L-serine (2.63g, 0.025 mole) were added to a reaction flask. Phosphoric acid (10 ml, 85 wt%) was added and the reaction was carried out at 155°C ($\pm 2^\circ\text{C}$) for up to 10 hours, preferably 6 hours. The molar ratio of aspartate: serine was 4:1 and the moles of H_3PO_4 added were from 0.01 to 0.1 preferably 0.02 to 0.04. The final product had no serine, and a mixture of alanine and

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dehydroalanine similar to the above product.

All thermal copolymers are purified after synthesis by dissolving them in distilled water and dialyzing them against same. These are then lyophilized for storage or,
5 in the case of example 1, chemically phosphorylated as follows.

Serine residues incorporated into thermal polymers were phosphorylated via the method of Neuhaus and Korkes (Neuhaus, F.C. and S. Korkes (1958). Phosphoserine.
10 Bioch. Prep., 6, 75-79) as reported by Sikes et al. (Sikes, C.S. Inhibition of mineral deposition by phosphorylated and related polyanionic peptides. U.S. Patent application 07/339,672). Phosphorus oxychloride, POCl₃, was added as 117 ml (1.25 moles) to 45 ml (2.5
15 moles) of water. This solution was stirred for one hour, allowing formation of monochlorophosphate (ClH₂PO₃). Next, amounts up to 0.25 moles of peptides were added with stirring and occasional heating at 60°C for two hours. The reaction was ended by dropwise addition of 18
20 ml (1 mole) of H₂O to degrade any unreacted monochlorophosphate to orthophosphate. Any polyphosphates that may have formed during the reaction were destroyed by addition of 75 ml of 1N HCl and heating in a boiling water bath for at least 10 minutes. Upon
25 cooling, peptides were crystallized in 95 percent ethanol and methyl butyl ether at 3°C overnight. The extent of phosphorylation of peptide was monitored spectrophotometrically upon formation of the phosphomolybdate complex (Eisenreich, S.J., R.T. Bannerman, D.E. Armstrong (1975). A simplified
30 phosphorus technique. Environ. Lett., 9, 43-53).

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Other useful derivatives are envisioned as well; for example, peptides containing sulfated, phosphonated, and sulfonated residues.

Serine residues incorporated into thermal polymers
5 were sulfated via the pyridinium acetyl sulfate (PAS)
method of Penke, et al. (Penke, B., et al. (1984).
Synthesis of potent heptapeptide analogues of
cholecystokinin. J. Med. Chem., 27, 845-849) as follows.
PAS was prepared by first mixing 28.4 ml of acetic
10 anhydride with 8.05 ml of pyridine at room temperature
with constant stirring. This mixture was cooled to -13°C
in an ice-methanol bath. To this solution 5.4 ml of
concentrated H₂SO₄ were added dropwise. Ether (300 ml)
was then added to precipitate the PAS. The precipitate
15 was filtered out and washed with three changes of ether.
The remaining PAS was air dried for three hours.

Sulfation was carried out by suspending 100 mg of
base hydrolyzed thermal polymer of example 1 in 10 ml of
pyridine with stirring. The peptide does not dissolve.
20 To this suspension, 54 mg of PAS were added. This was
stirred for 24 hours. The reaction mixture was then
diluted with 800 ml of distilled water and the pH
adjusted to 7.0 with 4 N NaOH. This was purified by
filtration followed by 4 washes with methanol. The
25 extent of sulfation was monitored using ion
chromatography. A 1 mg/ml solution of sulfated peptide
in 0.5 N NaOH was digested by autoclaving for 1 hour at
132°C and 30 PSI. This stock solution was then diluted
to 0.1 mg/ml with deionized water and 100 ml was injected
30 on an Ion Pac AS4A column (Dionex). The sample was
eluted at 1 ml/min with a buffer of 1.8 M Na₂CO₃/1.4 M

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NaHCO₃ and conductivity was measured with a Dionex conductivity detector.

Activity Assays:

To measure the ability of the peptides of the present invention to inhibit mineralization, a number of assays have been developed. These assays include the following:

1. pH-drift assay - CaCO₃.
2. pH-drift assay calcium phosphate.

The following examples describe how these assays have been employed to measure the ability of the polypeptides to inhibit mineralization.

Example 1: The pH-drift assay - Calcium Carbonate

A solution supersaturated with respect to CaCO₃ is prepared by separately pipetting 0.3 ml of 1.0 M CaCl₂ dehydrate and 0.6 ml of 0.4 M NaHCO₃ into 29.1 ml of artificial seawater (0.5 NaCl, 0.011 M KCl). The reaction vessel is a 50 ml, 3-necked, round-bottom flask partially immersed in a thermostated water bath at 20°C. The reaction vessel is closed to the atmosphere to minimize exchange of CO₂. The reaction is started by adjusting the pH upward to 8.3 by titration of μ l amounts of 1 N NaOH by digital pipette. The initial concentrations are 8 mM each of Ca²⁺ and 8 mM dissolved inorganic carbon (DIC). The reaction is monitored by pH electrode and recorded by strip chart.

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After a period of stable pH during which crystal nuclei form, the pH begins to drift downward until the reaction ceases due to depletion of reactants and the lowering of pH. The reaction may be quantified by
5 calculations based on DIC equilibria to give the amount of precipitation versus time. In Figure 1 it can be seen that a change in pH is directly proportional to a change in DIC from pH 8.3 to 7.7, but below 7.7 the buffering effect of the DIC system leads to greater changes in DIC
10 per unit pH.

In all experiments measuring inhibition of CaCO_3 crystallization by thermal polypeptides, peptides were added in solution at $0.05 \mu\text{g/ml}$. Note that high temperature polyaspartic acid shows increased activity
15 over the low temperature thermal polyaspartate (Table 1, Fig. 1). Also note the dramatic increase in activity after phosphorylation of the thermal PolyAsp:Asp:Ser molecules. The degree of inhibition of CaCO_3 formation is indicated by the length of time prior to
20 crystallization, shown by stabilization of pH of the metastable solution at about pH 8.3.

Example 2: The pH-drift assay: Calcium Phosphate

A solution supersaturated with respect to calcium phosphate is prepared by separately pipetting 0.1 ml of
25 1.32 M CaCl_2 dehydrate and 0.1 ml of $0.90 \text{ M NaH}_2\text{PO}_4$ into 29.8 ml of distilled water. This yields initial concentrations of 4.4 mM Ca^{2+} and 3.0 mM dissolved inorganic phosphorus (DIP). The reaction vessel is a 50 ml, round-bottom, 3-necked flask partially immersed in a
30 thermostated water bath at 20°C . The reaction vessel is

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closed to the atmosphere. The reaction begins upon mixing the reactants with an initial pH of 7.4.

Figure 2 shows data obtained from this type of assay. Amorphous calcium phosphate (ACP) nucleates immediately and slowly grows as indicated by a slight decrease in pH during the first 30 minutes or so of the assay. Following this, ACP begins to transform to calcium hydroxylapatite (HAP), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, as indicated by a marked acceleration in the downward pH drift. The reaction ceases as reactants are depleted and the pH is lowered.

In experiments measuring inhibition of calcium phosphate crystallization by thermal polypeptides, the concentration of peptides in solution was 30 $\mu\text{g/ml}$. The inhibitory activity of the peptides was shown by the degree to which they stabilized the downward pH-drift as calcium phosphate crystals grow. Similar increases in activity to those described for CaCO_3 were observed for calcium phosphate inhibition with the phosphorylated peptide clearly showing the best activity (Table 1, Fig. 2).

Uses of the Present Polypeptide

The various polypeptides of this invention may be utilized directly without additives or carriers for inhibiting the deposition of minerals such as phosphates and carbonates whether of inorganic or biological origin. Uses for inhibition of other salts of carbonate, phosphate and sulfate, e.g., magnesium or barium salts, are contemplated. The various polypeptides may be

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utilized by adding an effective amount of the inhibitor to a liquid in contact with a surface on which the deposits may form. Such is the case of industrially useful and commercially important containers, i.e.,
5 boilers, piping, desalinators, cooling towers, and the like. The various amino acid polymers of this invention can be added to water, water-containing liquids or other liquids in an amount as small as 0.01 ng/ml. The upper limit for the amount of the inhibitor is generally only
10 given by its solubility in the liquid to which it is added. However, if the presence of insoluble residues of these polymers does not interfere with industrial operations, it may be desirable to add these inhibitors in an amount greater than that given by their solubility
15 limit.

A preferred range of the various peptide derivatives for controlling inorganic scaling of, e.g., calcium carbonate is 10^{-4} - 10^2 $\mu\text{g/ml}$. Other preferred ranges are 10^{-4} -0.1 $\mu\text{g/ml}$ and 0.1- 10^2 $\mu\text{g/ml}$ of the various polymeric
20 derivatives.

When the present inhibitors are utilized for their antifouling characteristics in order to prevent the encrustations of plant or animal organisms, they can be added to a liquid such as water, water-containing liquids
25 or other non-aqueous liquids, preferably in an amount about 0.001-1,000 $\mu\text{g/ml}$; although larger amounts can also be used. Used within this range of concentrations, the present inhibitors find an application in the prevention of encrustation of organisms in, e.g., running water
30 piping or sewage piping, among others.

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The present inhibitors can also be applied directly to a surface before it comes in contact with mineral containing liquids, e.g., industrial containers, marine surfaces such as those in piers, ships, and the like.

5 The present inhibitors may be applied by themselves or in combination with other salt deposition inhibitors, antirust agents, or the like and/or with a carrier, directly to the exposed surface, or they may be mixed with other polymers used for the protection of said
10 surfaces. A variety of carriers are contemplated for the application of the present inhibitors. Some of the most common carriers include aqueous and non-aqueous liquids, gels, oils, organic and inorganic solvents, compressed gases, and the like. However, any carrier may be used
15 according to the needs. When used in high concentrations by themselves, the polyamino acid inhibitors of this invention may be highly viscous and can be easily applied to a surface.

After the application of the inhibitors, an
20 appropriate length of time may be allowed for the penetration of the inhibitor into the surface, as is the case with porous surface materials, such as wood, ceramics, and the like. Thus, a large storage of the present inhibitors is created within the material and the
25 surface may then be partially sealed with a coat-forming polymer to retard release of the active component.

Alternatively, the various polypeptides may be mixed with a carrier to form a suspension which can be applied to a surface. The present inhibitors may be applied to
30 any type of surface which may be exposed to the formation of inorganic or biological mineral deposits. Some of the

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most common materials to which the present inhibitors may be applied are metals, woods, synthetic polymers and copolymers, glass, ceramics, and painted to otherwise coated surfaces, although other materials are also contemplated. When in contact with the mineral-containing liquid, the inhibitors will slowly leach out from underneath the polymeric coating layer. The present inhibitors may further be applied in a mixture with the coating forming polymer, e.g., paints or any synthetic polymer used for the protection of surfaces such as polyurethanes. Alternatively, the coat-forming material may be an emulsion paint, etc. When the present inhibitors are used in admixtures with a coat-forming polymer or material, they can be used in a concentration of between 0.001-90% by weight of the total composition, although higher or lower concentrations are also contemplated in this invention. Some of the preferred concentrations are 1-75% by weight. Other preferred concentrations are 5-25%, 25-50% and 10-40% by weight. When applied to a surface the present inhibitors may be formulated with a carrier in the form of powder, solution, suspension, gel, oil, aerosol, paste or viscous colloid.

In a preferred embodiment of the present invention, the present materials may serve as inhibitors of dental tartar and plaque formation (referred to herein as tartar barrier agents) for human or animal use. In accordance with this embodiment of the present invention, the oral compositions may comprise any conventional pharmaceutically acceptable oral hygiene formulation that contains and is compatible with an effective amount of an antidental calculus agent as disclosed herein. Such

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formulations include, for example, mouthwashes, rinses, irrigating solutions, abrasive and non-abrasive gel dentifrices, denture cleansers, coated dental floss and interdental stimulator coatings, chewing gums, lozenges, 5 breath fresheners, foams and sprays. These formulations may be used to treat natural or artificial tooth enamel, or any orally compatible material which is subject to mineral deposition. Although human use is preferred, use in animals is also possible.

10 The tartar barrier agents may be present in the formulations in effective concentrations generally in the range of from about 0.05 wt.% to as much as 30 wt.% or the limit of compatibility with a vehicle. A preferred concentration range for the agents of the formulations of 15 the invention is from about 0.5 to about 10 wt.%. A more preferred range is from about 2 to about 8 wt.%.

The metal salts of the peptides of the present invention can be included in compositions up to the limit of their solubility therein, preferably around 0.2 to 2%, 20 with 10% being a reasonable maximum limit, by weight. In addition, the peptides of the present invention may be used in their acid forms, or as, for example, sodium salts, alone or in combination along with inorganic salts which are known by the prior art to be inhibitors of 25 mineralization. The combined percentage of these materials, which together operate as anticalculus agents, can be included in the composition in concentrations of from 0.2 to 10%, by weight, preferably around 2% by weight.

30 The pH of these preparations should be between about

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pH 5 and 10, preferably between pH 5 and 8, more preferably between about 6 and 7.5. A pH lower than 5 is undesirable because of the possible enhancement of enamel demineralization.

5 Suitable conventional pharmaceutically acceptable vehicles that can be employed with the tartar barrier agents to prepare the compositions of this invention may comprise water, ethanol; such humectants as polypropylene glycol, glycerol and sorbitol; such gelling agents as
10 cellulose derivatives, for example, Methocel carboxymethylcellulose (CMC 7MF) and Klucel HF, polyoxypropylene/polyoxyethylene block copolymers, for example, Pluronic F-127, Pluronic F-108, Pluronic P-103, Pluronic P-104, Pluronic P-105, and Pluronic P-123,
15 colloidal magnesium aluminosilicate complexes such as Veegum, and mucoprotein thickening agents such as Carbopol 934; gel stabilizers such as the silicon dioxides, for example Cab-O-Sil M5, and polyvinylpyrrolidone; sweeteners such as sodium saccharin and
20 aspartame; preservatives such as citric acid, sodium benzoate, cetylpyridinium chloride, potassium sorbate, methyl and ethyl parabens; detergents such as sodium lauryl sulfate, sodium cocomonoglyceride sulfonate, sodium lauryl sarcosinate and polyoxyethylene
25 isohexadecyl ether (Arlasolve 200) and approved colors and flavors.

The following specific examples will serve further to illustrate the tartar barrier agent compositions of this invention.

30 EXAMPLE A - Mouthwash Solution

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	Tartar barrier agent	0.5-2.0%
	w/w	
	Glycerol (humectant)	6.0
	Pluronic F-108	1.0
5	Sodium saccharin (sweetener)	0.3
	Deionized Water	q.s.
	Flavors	<u>1.0</u>
		100.0

EXAMPLE B - Mouthwash Solution

10	Tartar barrier agent	0.5-3.0%
	w/w	
	Ethanol, USP	15.0
	Pluronic F-108 (foaming agent)	2.0
	Glycerol (humectant)	10.0
15	Sorbitol (humectant)	10.0
	Sodium saccharin (sweetener)	0.2
	Deionized Water	q.s.
	Flavors	<u>0.2</u>
		100.0

20 EXAMPLE C - Abrasive Dentifrice Gel

	Tartar barrier agent	2.0-10.0%
	w/w	
	Fumed Silica (abrasive)	55.0
	Sodium Lauryl Sulfate (detergent)	1.5
25	Glycerol (humectant)	10.0
	Carboxymethylcellulose (gelling agent)	2.0
	Sodium saccharin (sweetener)	0.2
	Sorbitol (humectant)	10.0
	Flavors	1.0

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Deionized Water	q.s.
Preservative	<u>0.05</u>
	100.0

EXAMPLE D - Chewing Gum

5	Tartar barrier agent	1.0-11.0%
	w/w	
	Gum Base	21.3
	Sugar	48.5-58.5
	Corn Syrup (Baume 45)	18.2
10	Flavors	<u>1.0</u>
		100.0

EXAMPLE E - Nonabrasive Gel Dentifrice

	Tartar barrier agent	0.05-30.0%
	w/w	
15	Sorbistat (preservative)	0.15
	Deionized Water	q.s.
	Silicon Dioxide (gel stabilizer)	1.0
	Pluronic F-127 (gelling agent)	20.0
	Sodium saccharin	0.2
20	Flavors	<u>1.5</u>
		100.0

In addition to the above materials which can be included in the present tartar barrier compositions, it is also contemplated to include therein a protease inhibitor to prevent the present peptides and polypeptides from being degraded by various proteolytic enzymes.

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Examples of such inhibitors include aprotinin and trypsin inhibitor types I-P, I-S, II-L, II-O, II-S, IIT, III-O, and IV-O, although other inhibitors are within the scope of this invention. Similarly, when phosphopeptides
5 are employed, it is contemplated to use phosphatase inhibitors in conjunction with the polypeptide to prevent or inhibit dephosphorylation of the polypeptides. Examples of such phosphatase inhibitors are sodium fluoride, adenosine diphosphate, and vinyl ether/maleic
10 acid polymers (gantrez). Use of other phosphatase inhibitors is also possible.

The present peptides and polypeptides could also be linked to antibodies, or otherwise used in combination with antimicrobial agents, particularly those against
15 cavity-causing bacteria, as part of a tartar barrier composition to enhance antibacterial activity.

The polypeptides of the present invention may also find suitable use in treatment and prevention of mineral buildups in arteries and veins, such as in
20 atherosclerosis.

In this connection, the mode of administration of the polypeptides is preferably parenteral, i.e., intravenous, intraperitoneal, intramuscular, or subcutaneous, with intravenous administration being most
25 preferred. They may be administered alone, without a carrier vehicle; however, they may also be administered with pharmaceutically acceptable nontoxic carriers, the proportions of which are determined by the suitability and chemical nature of the particular carrier. For
30 intravenous or intramuscular administration, they may be

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used in the form of a sterile solution containing other solutes, for example, sufficient saline or glucose to make the solution isotonic. Like insulin, the peptides of the subject invention may also prove to be

5 administrable by use of a continuous perfusion device, which should simplify the method of administration.

The physician will determine the dosage which will be most suitable for a particular situation. Dosages will generally depend upon the age and size of the

10 patient, and the seriousness of the condition to be treated. A normal dosage will generally be in the range of 200-600 mg peptide per day.

The polypeptides of the present invention can also be used to impregnate prosthetic devices. For example,

15 the polypeptides of the present invention could be incorporated into polymeric based (e.g., a copolymer of ethylene - vinyl acetate or a silicon rubber) controlled released drug delivery matrices for site specific therapy directly into the perianular region of the heart

20 prosthesis (Levy, R.J. et al., CRC Critical Reviews in Biocompatibility 2, 148-187 (1986)). Controlled release devices incorporating a phosphonate derivative have been formulated to deliver that drug for more than 30 years without depletion. In addition, valve cusps could also

25 be preloaded with a polypeptide according to the present invention via covalent aldehyde-amino linkages; such an approach could be useful as a primary anti-calcium measure or as an adjunct for priming controlled release anti-mineralization therapy.

30 The invention now being fully described, it will be

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apparent to one of ordinary skill in the art that many changes and modifications can be made thereto, without departing from the spirit or scope of the invention as set forth therein.

Summary of Inhibitory Peptides

Reactants	Temp of Synthesis	Time of Synthesis	Amino Acid Analysis of Product Composition Exclusive of Asp	Inhibition CaCO ₃ /CaPO ₄ minutes	Approx. M.W. (GPC)
PolyAsp	190°C	72 hr		120 / 50	2000
	240°C	6 hr		220 / 240	6000**
		8 hr		230 / 279	6000**
Asp:Ser (4:1)	155°C	12 hr	2.4% Ser / 2.5% Ala; d-Ala*	/ 95	1100
		18 hr	6.6% Ser / 5.5% Ala; d-Ala	/ 94	1100
		24 hr	5.2% Ser / 3.4% Ala; d-Ala	/ 250	1100
		30 hr	7.2% Ser / 3.8% Ala; d-Ala	/ 410	1100
		36 hr	0.2% Ser / 0.5% Ala; d-Ala	/ 65	1100
		48 hr	0.3% Ser / 1.1% Ala; d-Ala	/ 75	1100
Asp:Ser (4:1) H ₃ PO ₄	155°C	6 hr	9.2% Ala; d-Ala	520 / 760	2400
PolyAsp:Asp:Pser H ₃ PO ₄	155°C	6 hr	0.5% Ser / 10% Ala; d-Ala	540 / 503	2200
PolyAsp:Asp:Pser (4:2:1) low-temp PolyAsp	155°C	9.5 hr	8.3% Ser / d-Ala present	790 / 1115	2500
PolyAsp:Asp:Pser (4:2:1) hi-temp PolyAsp	155°C	9 hr	8.8% Ser / 3.1% d-Ala	726 / 1240	2800
		18 hr	6.1% Ser / d-Ala present	393 / 810	2500
PolyAsp:Asp:Ser (4:2:1)		24 hr	6.7% Ser / d-Ala present	/ 390	2500

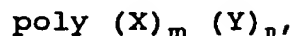
*d-Ala = dehydroalanine

** - Highly branched molecules. The apparent molecular weight may differ from actual, due to interference with the GPC system induced by branching. The system is calibrated for linear polyaspartates

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Claims

1. A polypeptide which inhibits mineral deposition, said polypeptide having the formula



5 wherein X is selected from the group consisting of aspartate, glutamate, glutamine, asparagine, mixtures and block polymers thereof,

Y is selected from the group consisting of phosphoserine, sulfoserine, phosphohomoserine,
10 phosphotyrosine, phosphothreonine, phosphoglutamine, phosphoasparagine, serine, alanine, dehydroalanine and mixtures thereof,

m = 5-199 and n = 1-40.

2. The polypeptide of Claim 1, wherein X is
15 aspartate or glutamate.

3. The polypeptide of Claim 2, wherein X is polyaspartate of 10-60 aspartate residues.

4. The polypeptide of Claim 1, wherein said polypeptide comprises 1-10 phosphoserines.

20 5. The polypeptide of Claim 4, wherein said polypeptide comprises 3-6 phosphoserine residues, 2-5 residues selected from the group consisting of sulfoserine, phosphohomoserine, phosphotyrosine,

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phosphothreonine, phosphoglutamine, phosphoasparagine, alanine, dehydroalanine, and mixtures thereof, and a block of 4-60 residues of polyaspartate.

6. The polypeptide of Claim 1, wherein said
5 polypeptide comprises 1-10 residues of alanine, 1-12 residues of an amino acid selected from the group consisting of phosphoserine, sulfoserine, phosphohomoserine, phosphotyrosine, phosphothreonine, phosphoglutamine, phosphoasparagine, alanine,
10 dehydroalanine, and mixtures thereof, 0-2 residues of serine, and the balance a block of 40-60 residues of polyaspartate.

7. The polypeptide of Claim 6, wherein said polypeptide comprises 3-6 residues of dehydroalanine.

15 8. The polypeptide of Claim 1, comprising 2-10 residues of a serine derivative selected from the group consisting of alanine, dehydroalanine and mixtures thereof, and 20-40 residues of aspartic acid.

9. The polypeptide of Claim 8, wherein said
20 polypeptide comprises 5-7 residues of said serine derivatives.

10. A method for the synthesis of a polypeptide of Claim 1, comprising combining amino acid residues corresponding to X, selected from the group consisting of
25 aspartate, glutamate, glutamine, asparagine and mixtures thereof, together with serine, in a reaction vessel, and heating said reaction vessel to a temperature sufficient to cause polymerization of said amino acid residues and

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below the decomposition temperature of serine, for up to 72 hours, until said amino acid residues polymerize.

11. The method of Claim 10, wherein said heating is conducted at 120°C-170°C.

5 12. The method of Claim 10, wherein said method comprising eliminating oxygen from said reaction vessel, to preclude charring.

10 13. The method of Claim 12, wherein said oxygen is eliminated by continuous purging of the reaction vessel with nitrogen.

14. The method of Claim 10, which further comprises combining in said reaction vessel prior to said polymerization reaction polyaspartate.

15 15. The method of Claim 14, wherein said polyaspartate is synthesized by heating aspartic acid in a reaction vessel maintained in a heating medium at about 160-190°C for a period of up to 72 hours until polymerization of said aspartic acid occurs.

20 16. The method of Claim 15, wherein oxygen is eliminated from said reaction vessel, to preclude charring.

17. The method of Claim 16, wherein oxygen is eliminated by continuous purging of the reaction vessel with a stream of nitrogen.

25 18. The method of Claim 15, wherein polyaspartate

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is synthesized by placing aspartic acid residues in an open reaction vessel and heating at a temperature of about 190-260°C for a period of up to 8 hours until said aspartic acid residues polymerize.

5 19. The method of Claim 10, further comprising adding phosphoric acid to said reaction vessel prior to polymerization.

20. The method of Claim 10, further comprising derivitizing serine residues in said polypeptide after
10 said polymerization, comprising phosphorylating, sulfating, phosphonating and sulfonating said serine residues.

21. A method of inhibiting mineral deposition on a surface, comprising contacting said surface with a
15 liquid, said liquid comprising the polypeptide of Claim 1 in a concentration no less than 0.01 ng/ml.

22. The method of Claim 21, wherein said concentration is 10^{-4} - 10^2 µg/ml.

23. A method of forming a mineral deposition
20 inhibiting coating on a surface, comprising coating said surface with a coat-forming polymer comprising the polypeptide of Claim 1 in a concentration of 0.001-90% by weight.

24. The method of Claim 23, wherein said
25 concentration is 25-75% by weight.

25. A method of inhibiting the formation of dental tartar and plaque in animals comprising exposing surfaces

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susceptible to said tartar and plaque formation to oral compositions comprising the polypeptide of Claim 1 in a pharmaceutically acceptable carrier.

26. The method of Claim 25, wherein the polypeptide
5 of Claim 1 is present in an amount of 0.05 wt/% - 30 wt/% of the total oral composition.

27. An oral composition for the inhibition of dental tartar and plaque, comprising the polypeptide of Claim 1, and a pharmaceutically acceptable carrier.

10 28. The composition of Claim 27, wherein said composition is a mouthwash.

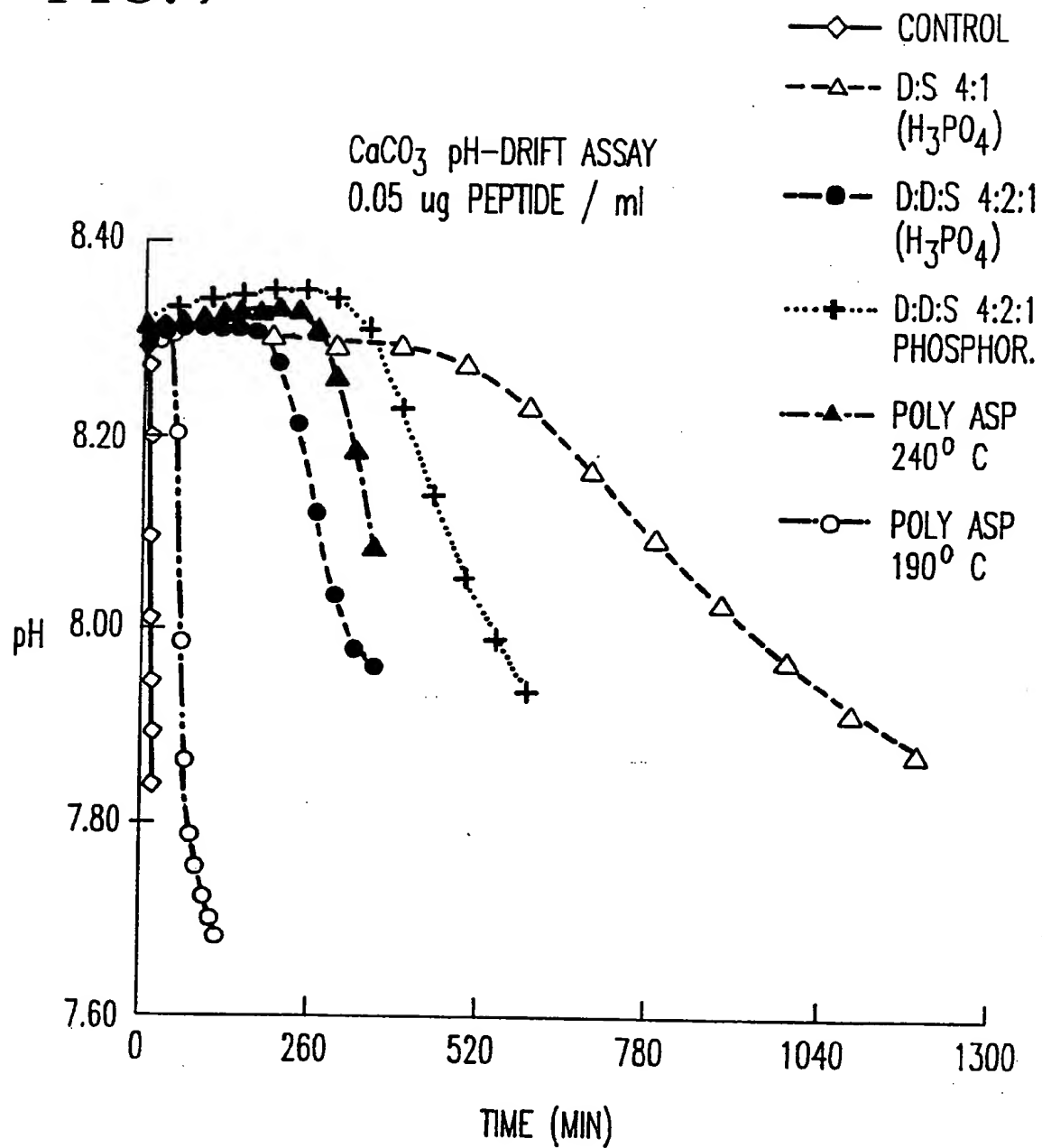
29. The composition of Claim 27, wherein said composition is a dentifrice.

15 30. The composition of Claim 27, wherein said composition is in the form of a chewing gum.

31. The composition of Claim 26, further comprising a pharmaceutically acceptable protease inhibitor.

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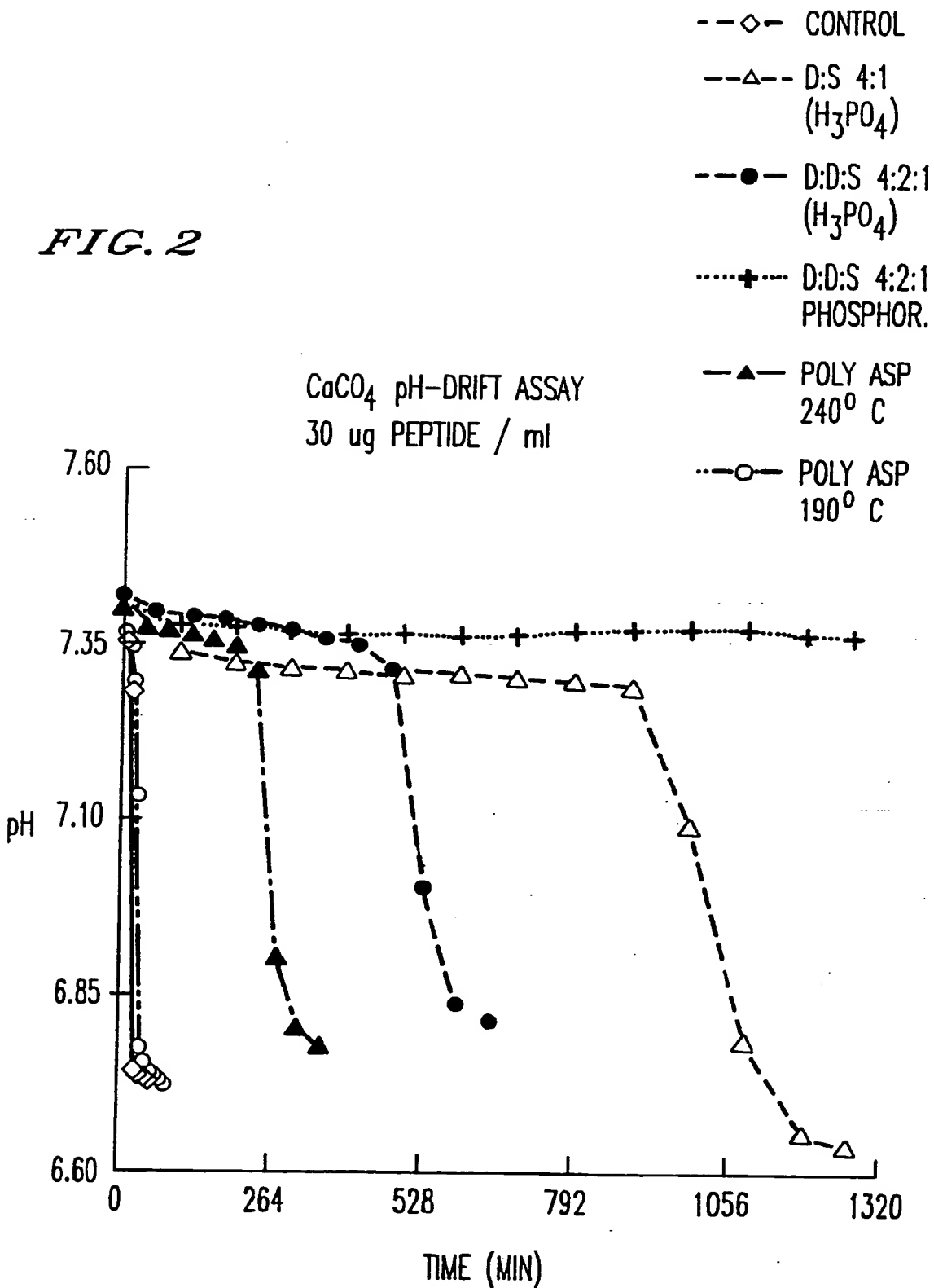
FIG. 1



SUBSTITUTE SHEET

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FIG. 2



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92.02433

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 37/02; C02F 5/10; C07K 7/06, 7/08, 7/10

US CL : Please See Attached Sheet.

II. FIELDS SEARCHED

Minimum Documentation Searched⁴

Classification System	Classification Symbols
U.S.	424/49, 54; 514/12, 13, 14, 15, 16, 17, 824, 901; 530/324, 325, 326, 327, 328, 329, 330; 427/384; 433/215

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴

Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	US, A, 4,866,161 (SIKES ET AL.) 12 September 1989. See entire document.	1-31
X	US, A, 4,868,287 (SIKES ET AL.) 19 September 1989. See entire document.	1-31
X,P	US, A 5,051,401 (SIKES ET AL.) 24 September 1991. See entire document.	1-31

* Special categories of cited documents:¹⁵

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search²

11 JUNE 1992

Date of Mailing of this International Search Report²

30 JUN 1992

International Searching Authority¹

ISA/US

Signature of Authorized Officer²⁰

LESTER L. LEE

(VSH)

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/49, 54; 514/12, 13, 14, 15, 16, 17, 824, 901; 530/324, 325, 326, 327, 328, 329,
330; 427/384; 433/215

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